Atty. Dkt. No. DOCKET_NO:TSRI 184.2C2

REMARKS

Claims:

After amending the claims as set forth above, claims 21, 32-41, 43-48, 53, 54, and 56-59 will be pending in this application.

Claim 21 as amended is directed to a plant comprising plant cells containing nucleic acid encoding a biologically functional immunoglobulin product comprising an immunoglobulin heavy chain polypeptide and an immunoglobulin light chain polypeptide wherein the nucleotide sequences also encode a leader sequence for each polypeptide and wherein the light chain polypeptide or the heavy chain prolypeptide or both are not full length. The amended claim further requires the plant cells to contain biologically functional immunoglobulin product encoded by the nucleotide sequences, wherein each leader sequence forms a secretion signal that is cleaved by proteolytic processing from each of the immunoglobulin heavy and light chain polypeptide.

Claim 43 as amended is directed to a plant, comprising plant cells containing nucleotide sequences each encoding an immunoglobulin single polypeptide product containing at least an immunoglobulin heavy chain polypeptide or portion thereof, an immunoglobulin light chain or portion thereof, or both an immunoglobulin heavy chain or portion thereof and an immunoglobulin light chain or portion thereof, wherein the nucleotide sequences encode a leader sequence forming a secretion signal. In addition, the claim excludes plant cells that contain nucleic acid encoding both a separate heavy and a separate light immunoglobulin chain. The claim further requires the plant cells to contain immunoglobulin single polypeptide product encoded by the nucleotide sequences, wherein the leader sequence is cleaved from said the polypeptide product following proteolytic processing.

Amendment Support

The amendments to claim 21 find ample basis in the application as filed. Support for plant cells containing nucleic acid encoding an immunoglobulin product comprising an immunoglobulin heavy chain polypeptide and an immunoglobulin light chain polypeptide

wherein the nucleotide sequences also encode a leader sequence for each polypeptide and wherein said light chain polypeptide or said heavy chain prolypeptide or both are not full length" can be found in the specification, for example as follows:

a) Page 11, lines 5-18, setting forth definitions of Fab fragment and Fv fragment. ("A Fab fragment may also be prepared by expressing in a suitable host cell the desired portions of immunoglobulin heavy chain and immunoglobulin light chain . . . .");

b) Page 15, lines 22 to 33, describing Fab and Fv fragments. ("fragment consisting of a portion of an immunoglobulin heavy chain and a portion of an immunoglobulin light chain.");

c) Page 16, lines 11 to 18, referring to an abzyme ("comprised of at least a portion of the immunoglobulin heavy chain variable region in association with another polypeptide chain, this other polypeptide chain includes at least the biologically active portion of an immunoglobulin light chain variable region.");

d) Page 28, lines 4-13, referring to transgenic plants containing an immunoglobulin product. ("Useful immunoglobulin products are well known to one skilled in the immunoglobulin art and include an immunoglobulin heavy chain, an immunoglobulin molecule comprised of a heavy and a light chain. One half of an immunoglobulin molecule, a Fab fragment, a Fv fragment, and proteins known as single chain antigen binding proteins.");

e) Page 31, line 26 to page 32, line 2 (emphasis added), referring to a biologically active glycopolypeptide multimer.

> ("[the multimer] comprises a polypeptide having a glycosylated core portion as well as N-acetylglucosamine containing outer branches and an amino acid residue sequence of an immunoglobulin molecule that is bonded to at least one other polypeptide . . . the other polypeptide may include an amino acid residue sequence of an immunoglobulin superfamily molecule, an immunoglobulin molecule, an immunoglobulin heavy chain, an immunoglobulin heavy chain variable region, **a portion of an immunoglobulin heavy chain variable region**, an immunoglobulin light chain, an immunoglobulin light chain variable region, or **a portion of an immunoglobulin light chain region**.").

Support for deletion of the phrase "not normally produced by the plant" in claim 21 can be found in the specification, for example, under the section entitled "SUMMARY OF THE INVENTION," which refers to sequences encoding immunoglobulins as "mammalian" sequences.

The amendments to claim 43 also find ample basis in the application as filed. Support for plant cells containing "nucleic acid encoding an immunoglobulin single polypeptide product containing at least an immunoglobulin heavy chain polypeptide or portion thereof, an immunoglobulin light chain or portion thereof" can be found in the specification, for example as follows:

a) Page 29, lines 28-29. ("In other preferred embodiments the immunoglobulins product consists of V_H **alone**, or of a V_H associated with a V_L to form a Fv fragment.") (emphasis added);

b) Page 30, lines 10-17, referring to an abzyme ("an abzyme constituted by either an immunoglobulin heavy chain and its associated variable region, or by an immunoglobulin heavy chain and an immunoglobulin light chain associated together to form an immunoglobulin molecule, a Fab, Fv or a substantial portion of an immunoglobulin molecule.");

c) Page 62 line 18 ("Example 2") to page 63, line 9, describing preparation of a plant expression vector encoding an immunoglobulin light chain single polypeptide with a leader sequence ("The resulting kappa light chain expression vector contained a gene coding for the entire kappa chain including the kappa leader.");

d) Page 64 line 18 ("Example 3") to page 65, line 5, describing preparation of a plant expression vector encoding an immunoglobulin heavy chain single polypeptide with a leader sequence ("The resulting gamma heavy chain expression vector contained a gene coding for the entire gamma heavy chain including the gamma leader.")

e) Page 68, Table 3 and accompanying text, describing immunological detection of gamma heavy chain and kappa light chain expressed separately as a

single polypeptide in a plant. (see numbers under headings "Gamma-L" and "Kappa-L").

f) Page 75, lines 5-13, describing detection of RNA encoding gamma heavy chain and kappa light chain expressed separately as a single polypeptide in a plant. (referring to kappa with leader and gamma with leader represented by lanes 3 and 4, respectively, in Figure 4 of U.S. 5,202,422);

g) Page 76, lines 22-32, describing immunological detection through Western blotting of gamma heavy chain and kappa light chain expressed separately as a single polypeptide in a plant. (referring to kappa with leader and gamma with leader represented by lanes 5 and 6, respectively, in Figure 5 of U.S. 5,202,422); and

h) See cited support under claim 21 above as support for "portions thereof," in claim 43.

Support for the amendment in claim 43 for nucleic acid encoding an immunoglobulin single polypeptide product containing "both an immunoglobulin heavy chain or portion thereof and an immunoglobulin light chain or portion thereof" can be found in the specification, for example as follows:

a) Page 10, lines 5-10, defining a single chain antigen-binding protein and encoding gene. ("A polypeptide composed of an immunoglobulin light-chain variable region amino acid sequence (V_L) tethered to an immunoglobulin heavy-chain variable region amino acid sequence (V_H) by a peptide that links the carboxyl terminus of the V_L sequence to the amino terminus of the V_H sequence.");

b) Page 28, lines 5-13, identifying useful immunoglobulin products in transgenic plants. ("and proteins known as single chain antigen binding proteins. . . . The structures of single chain antigen binding proteins has been described by Bird et al., Science, 242: 423-426 (1988) and U.S. Patent No. 4,704,692 to Ladner.").

In view of the above cited overwhelming support, it is respectfully submitted that the amendments raise no issue of new matter. Accordingly, entry of the amended claims into the case is respectfully requested.

AMENDMENT OF THE SPECIFICATION

In the previous amendment, Applicant requested to amend the specification to update the section entitled "Cross reference to Related Applications." The Office Action mailed July 5, 2001, entered the amendment with the exception of the phrase "the disclosures of which are incorporated by reference herein, " which was denied entry for allegedly constituting new matter. Applicant respectfully points out, however, that the Utility Patent Application Transmittal (copy attached as Exhibit A) that accompanied the instant patent application shows that box 5, requesting incorporation by reference of the parent application (08/642,456), was checked. It is further noted that the parent application (08/642,456) expressly incorporates by reference the applications that were referred to in the portion of Applicant's previous amendment that was denied entry. The relevant language from 08/642,456 is as follows:

> CROSS REFERENCE TO RELATED APPLICATION
>
> This is a continuation-in-part of U.S. application Ser. No. 07/971,951, filed Nov. 5, 1992 now U.S. Pat. No. 5,639,947, which is a continuation of U.S. Ser. No. 07/591,823, filed Oct. 2, 1990 (now U.S. Pat. No. 5,202,422), which is a continuation-in-part of U.S. Ser. No. 07/427,765, filed Oct. 27, 1989 (abandoned), the disclosures of which are incorporated by reference herein.

In view of the above, the Examiner is respectfully requested to withdraw the denial of entry and enter Applicant's prior requested amendment of the specification *in toto*.

DOUBLE PATENTING REJECTION

Claims 21, 32-54, 56-66 and 68-82 have been rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims

21-66 of copending Application No. 09/512,736. Claims 42, 49-52, 60-66 and 68-78 have been cancelled herein, thus rendering the rejection moot as to these claims. Although Applicant does not agree with the basis for this rejection, a terminal disclaimer that obviates the rejection is attached herewith. Applicant reserves the right to later withdraw the disclaimer depending on the claims that ultimately issue from copending Application No. 09/512,736.

REJECTION UNDER 35 U.S.C. § 102 OVER DÜRING

The rejection of claims 21, 32-39, 42-47, 49-54, 56, 57, 60-66, 68, 70-75, 78, and 80-82 under 35 U.S.C. § 102(b) as being allegedly anticipated by Düring (Dissertation) is respectfully traversed. Claims 42, 49-52, 60-66 and 68-78 have been cancelled herein, thus rendering the rejection moot as to these claims.

Claims 21 and dependents are not anticipated by Düring

With respect to claim 21 and its dependent claims, Düring fails to disclose or otherwise teach a plant comprising plant cells containing nucleic acid encoding an immunoglobulin heavy chain polypeptide and a light chain polypeptide wherein the light chain polypeptide or the heavy chain prolypeptide or both are not full length; Düring makes no mention of any immunoglobulin heavy or light chain that is not full-length. Encompassed by these claims are fragments of immunoglobulin such as those originally obtained by site specific proteolytic processing including the Fab'2 fragment and the Fab fragment, which comprise an assembled molecule which includes a full length light chain and a truncated heavy chain. Also encompassed are fragments originally generated primarily by non-proteolytic means such as the Fv fragment, which comprises both a truncated heavy and light chain.

Accordingly, on this basis alone, the rejection of claims 21 and its dependent claims under section 102(b) over Düring is without basis and should be withdrawn. It is further noted, however, that Düring also fails to disclose or otherwise teach the claimed requirement for a biologically functional immunoglobulin product encoded by the nucleotide sequences, wherein each leader sequence forms a secretion signal that is

cleaved from each of the immunoglobulin heavy chain and light chain polypeptide following proteolytic processing. The During thesis teaches an alternative strategy where the expression of the light chain is directed by a chimeric signal peptide-light chain gene which includes three amino acids not normally found at the C-terminus of a eukaryotic signal sequence (p. 18, line 7; Figure III/4). Using this strategy, Düring attempted to secrete a light chain only but ultimately failed to produce a detectable level of the polypeptide (Düring translation, p.80, line 2). During's failure to produce cells expressing only the light chain was likely a result of incorrect proteolytic processing of the leader from the light chain and failure of the protein to enter the eukaryotic secretory pathway (DECLARATION by Steven Mayfield under 37 C.F.R. § 1.132, dated May 13, 2001).

Accordingly, because Düring fails to disclose each and every element of the claimed invention, the rejection of claim 21 and its dependent claims under section 102(b) fails as a matter of law. The examiner, therefore, is respectfully urged to withdraw the rejection of the claims.

Claims 43 and dependents are not anticipated by Düring

With respect to claim 43 and its dependent claims, Düring fails to disclose or otherwise teach a plant comprising plant cells containing nucleic acid encoding an immunoglobulin single polypeptide product containing at least an immunoglobulin heavy chain polypeptide or portion thereof, an immunoglobulin light chain or portion thereof, or both an immunoglobulin heavy chain or portion thereof and an immunoglobulin light chain or portion thereof, wherein the nucleotide sequences encode a leader sequence forming a secretion signal wherein the cells containing nucleotide sequence encoding an immunoglobulin heavy chain polypeptide or portion thereof do not contain nucleotide sequence encoding a light chain, while the cells containing nucleotide sequence encoding an immunoglobulin light chain polypeptide or portion thereof do not contain nucleotide sequence encoding a heavy chain. Düring does not disclose or otherwise teach to express any immunoglobulin light or heavy chain polypeptide by itself. As already described, Düring tried but failed to express a light chain polypeptide without the heavy chain and Düring made no attempt to express a heavy chain polypeptide without a light chain

polypeptide. Düring also fails to disclose or otherwise teach plant cells containing nucleic acid express both a light and a heavy chain together as a single immunoglobulin polypeptide.

Accordingly, because Düring fails to disclose each and every element of the claimed invention, the rejection of claim 43 and its dependent claims under section 102(b) fails as a matter of law. The examiner, therefore, is respectfully urged to withdraw the rejection of the claims.

REJECTION UNDER 35 U.S.C. § 102 OVER GOODMAN

The rejection of claims 21, 32-40, 42-47, 49-54, 56-58, 60-66, 68, 70-76, and 78-82 under 35 U.S.C. § 102(e) as being allegedly anticipated by Goodman (U.S. No. 4,956,282) is respectfully traversed. Claims 42, 49-52, 60-66 and 68-78 have been cancelled herein, thus rendering the rejection moot as to these claims.

Claim 21 and its dependents are not anticipated by Goodman

With respect to claim 21 and its dependent claims, Goodman fails to disclose or otherwise teach a plant comprising plant cells containing nucleic acid encoding an immunoglobulin heavy chain polypeptide and a light chain polypeptide wherein the light chain polypeptide or the heavy chain polypeptide or both are not full length. This is readily evident because Goodman makes only a passing reference to expressing immunoglobulins, which is nearly buried amidst a laundry list of known mammalian proteins as seen below:

> Structural genes of interest include .alpha.-, .beta.- and .gamma.-interferons, **immunoglobulins, with the structural genes coding for the light and heavy chains and desirably assembly occurring in the plant cell**, lymphokines, such as interleukins 1, 2 and 3, growth factors, including insulin-like growth factor, epidermal growth factor, platelet derived growth factor, transforming growth factor-.alpha., -.beta., etc., growth hormone, insulin, collagen plasminogen activator, blood factors, such as factors I to XII, histocompatibility antigens, enzymes, or other mammalian proteins, particularly human proteins.

U.S. No. 4956282, col. 3, lines 11-30 (emphasis added). There is clearly nothing in this statement that refers directly or indirectly to an immunoglobulin with less than a full length heavy or light chain.

Accordingly, because Goodman fails to disclose each and every element of the claimed invention, the rejection of claim 21 and its dependent claims under section 102(e) fails as a matter of law. The examiner, therefore, is respectfully urged to withdraw the rejection of the claims.

Claim 43 and its dependents are not anticipated by Goodman

With respect to claim 43 and its dependent claims, Goodman fails to disclose or otherwise teach a plant comprising plant cells containing nucleic acid encoding an immunoglobulin single polypeptide product containing at least an immunoglobulin heavy chain polypeptide or portion thereof, an immunoglobulin light chain or portion thereof, or both an immunoglobulin heavy chain or portion thereof and an immunoglobulin light chain or portion thereof, wherein the nucleotide sequences encode a leader sequence forming a secretion signal wherein the cells containing nucleotide sequence encoding an immunoglobulin heavy chain polypeptide or portion thereof do not contain nucleotide sequence encoding a light chain, while the cells containing nucleotide sequence encoding an immunoglobulin light chain polypeptide or portion thereof do not contain nucleotide sequence encoding a heavy chain.

Again, the limited description in Goodman concerning antibody expression makes no mention whatsoever to expressing immunoglobulin single polypeptides of any form. Accordingly, because Goodman fails to disclose each and every element of the claimed invention, the rejection of claim 43 and its dependent claims under section 102(e) fails as a matter of law. The examiner, therefore, is respectfully urged to withdraw the rejection of the claims.

REJECTION UNDER 35 U.S.C. § 103 OVER DÜRING

The rejection of claims 21, 32-54, 56-66 and 68-82 under 35 U.S.C. § 103(a) as being allegedly unpatentable over Düring in view of Applicant's allegedly admitted prior art is respectively traversed. Claims 42, 49-52, 60-66 and 68-78 have been cancelled herein, thus rendering the rejection moot as to these claims.

Relevant Law

A claimed invention is obvious if the differences between it and the prior art "are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." 35 U.S.C. § 103 (1994); see also Graham v. John Deere, 383 U.S. 1, 13 (1966). Federal Circuit case law provides that "[t]he consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in the light of the prior art." In re Dow Chem., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed.Cir.1988). Under the law, there must be a showing of a suggestion, teaching, or motivation to combine the prior art references is an "essential evidentiary component of an obviousness holding." C.R. Bard, Inc. v. M3 Sys. Inc., 157 F.3d 1340, 1352, 48 USPQ2d 1225, 1232 (Fed.Cir.1998). Also required is that the combined teachings have a reasonable expectation of success, viewed in light of the prior art. *See* In re Dow Chemical Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed.Cir.1988)("Both the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure.").

The examiner bears the burden of establishing a *prima facie* case of obviousness. In re Rijckaert, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed.Cir.1993); In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed.Cir.1992). This showing must be clear and particular, and broad conclusory statements about the teaching of multiple references, standing alone, are not "evidence." *See* Dembiczak, 175 F.3d at 1000, 50 USPQ2d at 1617. However, the suggestion to combine need not be express and "may come from the prior art, as filtered through the knowledge of one skilled in the art." Motorola, Inc. v. Interdigital Technology Corp., 121 F.3d 1461, 1472, 43 USPQ2d 1481, 1489 (F d.Cir.1997). Only when the examiner's burden is met does the burden of

coming forward with rebuttal argument or evidence shift to applicant. Rijckaert, 9 F.3d at 1532, 28 USPQ2d at 1956.

Claim 21 and its dependents are not obvious over Düring

To reiterate, claim 21 and its dependents require that plant cells contain nucleotide sequences encoding a biologically functional immunoglobulin product comprising an immunoglobulin heavy chain polypeptide and an immunoglobulin light chain polypeptide wherein said nucleotide sequences also encode a leader sequence for each polypeptide and wherein said light chain polypeptide or said heavy chain prolypeptide or both are not full length. Also required is that the plant cells contain the encoded biologically functional immunoglobulin product and that each leader sequence form a secretion signal which is cleaved by proteolytic processing from the immunoglobulin heavy chain and the light chain.

As already discussed under anticipation, the Düring dissertation fails to teach the requirement for nucleic acid encoding a biologically functional immunoglobulin product wherein the nucleic acid encodes a leader sequence for each polypeptide and wherein the light chain polypeptide or said heavy chain polypeptide, or both, are not full length. In particular, the strategy used by Düring for expression is different from Applicant's claimed invention. This is because Düring chose to add polar amino acids and a methionine between the mature variable light chain amino terminus and the leader peptide sequence. As previously argued by Applicant, Düring's strategy likely obscured the recognition site for proteolytic processing and removal of the leader peptide, a step that is necessary for proper assembly of a heavy-light chain antibody construct. Stephen Mayfield Declaration, page 2, ¶¶4-6. Thus, Düring fails to teach not only the claimed nucleic acid requirement that encodes less than a full length polypeptide, but also fails to teach the requirement for biologically functional immunoglobulin product where the leader from each chain is cleaved by proteolytic processing.

It is respectfully submitted, therefore, that the above noted deficiencies in the teachings of Düring with respect to claim 21 and its dependents clearly demonstrate that no substantive foundation exists upon which to find the claims obvious over this reference. Although the claims are not presently rejected as obvious over Düring in

combination with any specified prior art teaching, it is also submitted that no such teachings or combination of teachings exist that could cure the deficiencies noted for Düring.

The only other reference cited for obviousness in this action is Goodman, but this reference is similarly deficient in teaching or suggesting a heavy-light chain immunoglobulin wherein at least one of the chains are not full length. Goodman's teachings at best are limited to expressing gamma interferon, a lymphokine that in structurally and functionally distinct from immunoglobulin. Accordingly, the examiner is respectfully urged to withdraw the rejection of claims 21 and dependents as allegedly obvious over Düring.

Claim 43 and its dependents are not obvious over Düring

To reiterate, claim 43 and its dependents require plant cells containing nucleotide sequences each encoding an immunoglobulin single polypeptide product containing at least an immunoglobulin heavy chain polypeptide or portion thereof, an immunoglobulin light chain or portion thereof, or both an immunoglobulin heavy chain or portion thereof and an immunoglobulin light chain or portion thereof, wherein the nucleotide sequences encode a leader sequence forming a secretion signal. The claim further requires the plant cells to contain immunoglobulin single polypeptide product encoded by the nucleotide sequences, wherein the leader sequence is cleaved from said the polypeptide product following proteolytic processing. Expressly excluded from the claims are plant cells that contain nucleic acid encoding both a separate heavy and a separate light immunoglobulin chain.

As already discussed under anticipation, Düring fails to disclose or otherwise teach a plant comprising plant cells containing nucleic acid encoding any immunoglobulin single polypeptide product wherein the plant cells do not contain nucleic acid encoding both a separate heavy and a separate light immunoglobulin chain. In fact, if one takes the teachings of Düring at face value (ignoring what Applicant believes are significant deficiencies in enablement), Düring in fact teaches away from the claimed invention because Düring attempted and fail d to express an immunoglobulin light chain

polypeptide. Düring translation, p.80, line 2. Düring never attempted nor even discussed expression of the immunoglobulin heavy chain polypeptide by itself. Düring also never attempted or discussed expressing a single polypeptide heavy and light chain construct, such as a single chain Fv.

It is respectfully submitted, therefore, that the above noted deficiencies in the teachings of Düring with respect to claim 43 and its dependents clearly demonstrate that no substantive foundation exists upon which to find the claims obvious over this reference. Although the claims have not presently been rejected as obvious over Düring in combination with any specified prior art teaching, it is also submitted that no such teachings or combination of teachings exist that could cure the deficiencies noted for Düring.

The only other reference raised for obviousness in this action is Goodman, but the teachings of this reference are similarly deficient to that of Düring. When it comes to immunoglobulins, Goodman only mentions expressing immunoglobulin heavy and light chains together in the same cell so that the chains can assemble. Such immunoglobulin, however, does clearly does not teach or suggest the invention of claim 43 and its dependents. It can be argued that Goodman's failure to mention expression of an immunoglobulin heavy chain alone or light chain alone teaches constitutes a teaching away from the claimed invention (Goodman's failure to mention a single polypeptide that encodes both an immunoglobulin light and heavy chain is understandable since such construct was not known until years later). Accordingly, the examiner is respectfully urged to withdraw the rejection of claims 43 and dependents as allegedly obvious over Düring.

REJECTION UNDER 35 U.S.C. § 103 OVER GOODMAN

The rejection of claims 21, 32-54, 56-66 and 68-82 under 35 U.S.C. § 103(a) as being allegedly unpatentable over Goodman in view of Applicant's allegedly admitted prior art is respectively traversed. Claims 42, 49-52, 60-66 and 68-78 have been cancelled herein, thus rendering the rejection moot as to these claims.

Claim 21 and its dependents are not obvious over Goodman

As already mentioned, Goodman fails to mention a heavy-light chain immunoglobulin wherein at least one of the chains is not full length. Goodman's passing reference to expressing immunoglobulins is nothing more than an invitation to experiment. Goodman does not provide sufficient guidance to enable one skilled in the art to use methods for the production of a full-length immunoglobulin, let alone an immunoglobulin with a heavy or light chain (or both) that is not full length, because Goodman does not describe procedures for production of any immunoglobulin product whatsoever. Goodman is unaware of requirements for production of any immunoglobulin product in plants.

Furthermore, Goodman's teachings on gamma interferon expression would not reasonably have been considered to advance the possibility of immunoglobulin expression in plants. As already mentioned, gamma interferon is structurally and functionally distinct from immunoglobulin light or heavy chains, the latter of which are immunoglobulin superfamily members. Also, it was known by the late 1980s that antibodies encompassing a heavy and a light chain are secreted through a complex interaction between the chains and other proteins. For example, heavy chain production in B cells was known to precede that of light chain production in ontogeny, and that the heavy chain binds to the BiP protein in the endoplasmic reticulum before heavy chain assembles with light chain. See e.g., Hass et al., Proc. Natl. Acad. Sci. USA 81:7185-7188 (1984) (copy attached as Exhibit B, p.7187, right column, citing reference 11, Burrous et al.. PNAS USA 78:564 (1981)). It was also known that heavy chain production in the absence of light chain production was often fatal in mature B lymphocytes. See e.g., *Id.* (Exhibit B; p.7185, left column). Furthermore, if heavy chain were produced without light chains, the heavy chains were not secreted (see Pepe et al., J. Immunol. 137:2367-2372 (1986); copy attached as Exhibit C, p.2367, left column); The opposite was true, however, when light chains were secreted without heavy chains.

Thus, the absence of a chemical or biological relationship between gamma interferon and immunoglobulin, as well as the known complexity underlying immunoglobulin secretion would have rendered the teachings of Goodman largely irrelevant to the claimed invention. Furthermore, as already discussed, the teachings of

Düring do not in any way cure the deficiencies in the teachings of Goodman. Accordingly, the examiner is respectfully urged to withdraw the rejection of claims 21 and dependents as allegedly obvious over Goodman.

Claim 43 and its Dependents are not obvious over Goodman

Goodman's mere passing reference to immunoglobulins describes immunoglobulin heavy and light chains expressed together in the same cell. As already discussed, the absence of any mention in Goodman of an immunoglobulin single polypeptide product arguably constitutes a teaching away of the invention of claims 43 and dependents. Furthermore, Goodman also fails to mention a single polypeptide that encodes both an immunoglobulin light and heavy chain.

Furthermore, Goodman's teachings on gamma interferon expression would not reasonably have been considered to advance the possibility of expressing a single polypeptide heavy or light chain (or both) in plants. Gamma interferon is structurally and functionally distinct from an immunoglobulin light or heavy chain. Furthermore, it was well known that heavy chains expressed in the absence of light chains were generally toxic to B cells. See e.g., Hass et al., Proc. Natl. Acad. Sci. USA 81:7185-7188 (1984) (copy attached as Exhibit B, p.7187, right column, citing reference 11, Burrous et al., PNAS USA 78:564 (1981)). In view of this evidence, it is submitted that one of ordinary skill would have been surprised to discover that plant cells would be capable of supporting secretion of an immunoglobulin heavy chain alone, an immunoglobulin light chain alone or the two combined into a single polypeptide as required by claim 43 or its dependents. If this were not the case, then the present inventors' work simply would not have been roundly hailed as a breakthrough in the scientific literature and in the general press. See e.g., cover of Nature, 242(6245), 76-78, 1989 (copy attached as Exhibit D); Excerpt of article in the Los Angles Times (San Diego County), November 2, 1989 (copy attached as Exhibit E).

It is further noted, that assuming *arguendo* that Goodman's limited teachings support to some extent expression of an immunoglobulin single polypeptide in plants, any such teachings would be negated by Düring's attempt and failure to achieve this result for light chains (i.e., Düring translation, p.80, line 2). Accordingly, the examiner is

respectfully urged to withdraw the rejection of claims 43 and dependents as allegedly obvious over Goodman.

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is urged to contact the undersigned by telephone to address any outstanding issues standing in the way of an allowance.

Respectfully submitted,

Date: December 27, 2001

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Atty. Dkt. No. DOCKET_NO:TSRI 184.2C2

VERSION WITH MARKINGS TO SHOW CHANGES MADE

21. (Amended three times) A plant, comprising:

a) plant cells containing nucleotide sequences encoding [~~one or more~~] a biologically functional immunoglobulin product comprising an immunoglobulin heavy chain polypeptide and an immunoglobulin light chain polypeptide wherein said nucleotide sequences also encode a leader sequence for each polypeptide and wherein said light chain polypeptide or said heavy chain polypeptide or both are not full length [~~not normally produced by the plant~~]; and

b) biologically functional immunoglobulin product encoded by said nucleotide sequences, wherein each [~~nucleotide sequence encoding an immunoglobulin polypeptide encodes a~~] leader sequence [~~forming~~] forms a secretion signal that is cleaved from each of said immunoglobulin heavy chain and light chain polypeptide following proteolytic processing.

43 (Twice amended) A plant, comprising:

a) plant cells containing nucleotide sequences each encoding an immunoglobulin single polypeptide product containing at least [~~a portion of~~]an immunoglobulin heavy chain polypeptide or portion thereof, an immunoglobulin light chain or portion thereof, or both an immunoglobulin heavy chain or portion thereof and an immunoglobulin light chain or portion thereof, wherein said nucleotide sequences encode [~~polypeptide and polypeptide further comprises~~] a leader sequence forming a secretion signal wherein said cells containing nucleotide sequence encoding an immunoglobulin heavy chain polypeptide or portion thereof do not contain nucleotide sequence encoding a light chain, while said cells containing nucleotide sequence encoding an immunoglobulin light chain polypeptide or portion thereof do not contain nucleotide sequence encoding a heavy chain; and

b) [~~biologically functional~~] immunoglobulin single polypeptide product encoded by said nucleotide sequences, wherein said leader sequence is cleaved from said [~~heavy chain~~] polypeptide product following proteolytic processing.

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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	TSRI 184.2Cont
First Inventor or Application Identifier	Hein
Title	TRANSGENIC PLANTS EXPRESSING...
Express Mail Label No.	EL073302829US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. [X] * Fee Transmittal Form (e.g., PTO/SB/17) (Submit an original and a duplicate for fee processing)
2. [X] Specification [Total Pages 125]
   (preferred arrangement set forth below)
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings (if filed)
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) (35 U.S.C. 113) [Total Sheets 8]
4. Oath or Declaration [Total Pages 4]
   a. [ ] Newly executed (original or copy)
   b. [X] Copy from a prior application (37 C.F.R. § 1.63(d)) (for continuation/divisional with Box 17 completed) [Note Box 5 below]
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).
5. [X] Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. [ ] Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
   a. [ ] Computer Readable Copy
   b. [ ] Paper Copy (identical to computer copy)
   c. [ ] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [ ] Assignment Papers (cover sheet & document(s))
9. [ ] 37 C.F.R.§3.73(b) Statement (when there is an assignee) [ ] Power of Attorney
10. [ ] English Translation Document (if applicable)
11. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
12. [X] Preliminary Amendment
13. [X] Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
14. [ ] * Small Entity Statement(s) (PTO/SB/09-12) [ ] Statement filed in prior application, Status still proper and desired
15. [ ] Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. [X] Other: Certificate of Express Mail

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17. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:
    [X] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No: 08/ /642,406
    Prior application information: Examiner J. Haas Group / Art Unit 1649

18. CORRESPONDENCE ADDRESS

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Signature		Date	11/25/98

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Approved for use through 9/30/2000. OMB 0651-0032
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Patent fees are subject to annual revision on October 1. These are the fees effective October 1, 1997.
Small Entity payments must be supported by a small entity statement, otherwise large entity fees must be paid. See Forms PTO/SB/09-12.
See 37 C.F.R. §§ 1.27 and 1.28.

TOTAL AMOUNT OF PAYMENT ($) 2,398.00

Complete if Known	
Application Number	
Filing Date	November 25, 1998
First Named Inventor	Hein
Examiner Name	
Group / Art Unit	
Attorney Docket No.	TSRI 184.2Conl

METHOD OF PAYMENT (check one)

1. [X] The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:

Deposit Account Number: 19-0962
Deposit Account Name:

[X] Charge Any Additional Fee Required Under 37 C.F.R. §§ 1.16 and 1.17
[ ] Charge the Issue Fee Set in 37 C.F.R. § 1.18 at the Mailing of the Notice of Allowance

2. [X] Payment Enclosed: [X] Check [ ] Money Order [ ] Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	760	201	395	Utility filing fee	760
106	330	206	165	Design filing fee	
107	540	207	270	Plant filing fee	
108	790	208	395	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) ($) 760.00

2. EXTRA CLAIM FEES

	Extra Claims		Fee from below	Fee Paid
Total Claims	59 - 20** =	39	X 18	= 702
Independent Claims	15 - 3** =	3	X 12	= 936
Multiple Dependent				=

** or number previously paid, if greater; For Reissues, see below

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	11	Claims in excess of 20
102	78	202	41	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim, if not paid
109	82	209	41	** Reissue independent claims over original patent
110	22	210	11	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 1,638.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	950	217	475	Extension for reply within third month	
118	1,510	218	755	Extension for reply within fourth month	
128	2,060	228	1,030	Extension for reply within fifth month	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,320	241	660	Petition to revive - unintentional	
142	1,320	242	660	Utility issue fee (or reissue)	
143	480	243	225	Design issue fee	
144	670	244	335	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	790	246	395	Filing a submission after final rejection (37 CFR 1.129(a))	
148	790	248	395	For each additional invention to be examined (37 CFR 1.129(b))	
				Other fee (specify)	
				Other fee (specify)	

* Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

SUBMITTED BY				Complete (if applicable)	
Typed or Printed Name	Thomas Fitting			Reg. Number	34,163
Signature	Thomas Fitting	Date	11/25/98	Deposit Account User ID	

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Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7185–7188, November 1984
Immunology

Immunoglobulin heavy chain toxicity in plasma cells is neutralized by fusion to pre-B cells

(immunoglobulin chain loss/isoelectric focusing of heavy chain)

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Communicated by Niels K. Jerne, July 9, 1984

ABSTRACT **A plasma cell hybridoma frequently loses its immunoglobulin heavy (H) chain spontaneously but rarely is production of its light (L) chain lost. Upon fusion to a pre-B-cell hybridoma that produces no Ig chain, the L chain is frequently lost. In cells without the L chain the H chain, which is derived from the plasma cell, is not chemically modified. Our results indicate that, in pre-B cells, but not in plasma cells, there must be a mechanism that neutralizes the toxic effect of free H chain.**

Myelomas and hybridomas derived from plasma cells secrete immunoglobulin consisting of heavy (H) and light (L) chains. From such cell lines, subclones that have lost H chain expression can be recovered, but they still secrete the L chain (1, 2). The L chain can then be lost at the same frequency as the H chain. On the other hand, it seems to be very difficult to recover cells that have lost L chain expression but that still synthesize the H chain (1–3) except when the cells are mutagenized (4) or express a mutant H chain (5–7). These observations have led to the view that the free H chain is toxic to the cells (2, 3). However, cells of an earlier differentiation stage, pre-B cells, synthesize intracellular H chain in the absence of the L chain (8). To explain this difference, several possibilities have been proposed: (*i*) the rate of H chain synthesis in pre-B cells is too low to damage the cell (9); (*ii*) the pre-B cell synthesizes a different H chain that is not toxic to the cell (2); or (*iii*) in pre-B cells, there is a special protein that neutralizes the toxic effect of the free H chain (10, 11).

Here we report that hybridomas derived from pre-B cells are not different from plasma cell hybridomas with respect to their rate of H chain synthesis and steady-state level of H chain. However, they do not synthesize the L chain. In consequence, we wanted to answer the question whether there are pre-B-cell hybridomas that can synthesize a free H chain, which has been shown to be toxic in plasma cell hybridomas.

MATERIALS AND METHODS

Cell Lines. Sp2 and GK14.1 were established and provided by G. Köhler (Basel). The cell lines are derived from fusions between spleen cells and a myeloma, X63 Ag8, and synthesize IgG2b. Sp2.0 is an azaguanine-resistant subclone of Sp2 and has lost Ig expression. Cell lines H32-21, H32-3, H32-8, and H6 are derived from fusions between γ2b-synthesizing subclones of the Abelson virus-transformed pre-B-cell line 18-81 and X63 Ag8653 (12). Clone H62 is a subclone of H6 and synthesizes no Ig chain. NORA hybridomas were made by fusion of Sp2 HL Ag14 and H62. SPSP hybridomas are derived from a fusion between Sp2.0 and Sp2. Cell fusion was carried out as described (12). The genealogy of the various hybridomas is given in Fig. 1.

Isolation of Subclones with Ig Chain Loss. Soft agar cloning and antiserum overlay was carried out according to the method of Coffino and Scharff (1). When an antiserum against IgG2b (γ2b,κ) was used, about 1% of the cells formed colonies without precipitation. These clones were isolated, and their Ig expression was analyzed by immunofluorescence. Clones of interest were grown in mass culture and further analyzed by immunoprecipitation and electrophoresis.

$NaDodSO_4$/Polyacrylamide Electrophoresis. Cell labeling, immunoprecipitation, and $NaDodSO_4$/polyacrylamide gel electrophoresis were carried out as described (12). Pulse labeling (30 min) was carried out by the addition of [^{35}S]methionine to cells that had been incubated in methionine-free select medium/10% dialyzed fetal calf serum for 1 hr. The amount of [^{35}S]methionine incorporated into the precipitated proteins was measured after gel fractionation by scintillation assay of the solubilized gel slices.

Isoelectric Focusing. [^{35}S]Methionine-labeled Ig precipitates were dissolved in 9.5 M urea/2% Nonidet P-40/2% Ampholine (pH 5–11)/5% 2-mercaptoethanol and applied to isoelectrofocusing slab gels. The gel composition was according to O'Farrell (13). Electrophoresis was carried out for 1 hr at 250 V, for 12 hr at 400 V, and finally for 1 hr at 800 V. The proteins were visualized by fluorography.

Immunofluorescence. The purification and fluorochrome conjugation of goat antibodies specific for mouse H chain isotypes and the methods for immunofluorescence detection of intracellular Ig have been described (14).

RESULTS

The Rate of Ig H Chain Synthesis and the Steady-State Level of the H Chain in Plasma Cell- and in Pre-B Cell-Derived Hybridomas Are of the Same Order of Magnitude. The pre-B cell is characterized by the synthesis of intracellular H chain in the absence of the L chain. The free H chain is toxic in plasma cells, but pre-B cells may survive because they synthesize the H chain in small amounts (15, 16) that would not damage the cell. To test this hypothesis, we increased the amount of free H chain present in pre-B cells by fusion to a myeloma. The steady-state level of RNA specific for the H chain is the same in pre-B-cell hybridomas and in plasma cell hybridomas (16).

By measuring the short-term incorporation of radiolabeled methionine into the H chain (Fig. 2), we have compared the rate of Ig H chain synthesis in pre-B-cell-derived hybridomas with that of plasma cell-derived hybridomas. The amount of radioactivity incorporated into the various H chains was of the same order of magnitude. Also, the steady-state level of the H chain, as determined by long-term label-

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Abbreviations: H chain and L chain, heavy and light chain, respectively, of Ig; BiP, H chain binding protein.

EXHIBIT B

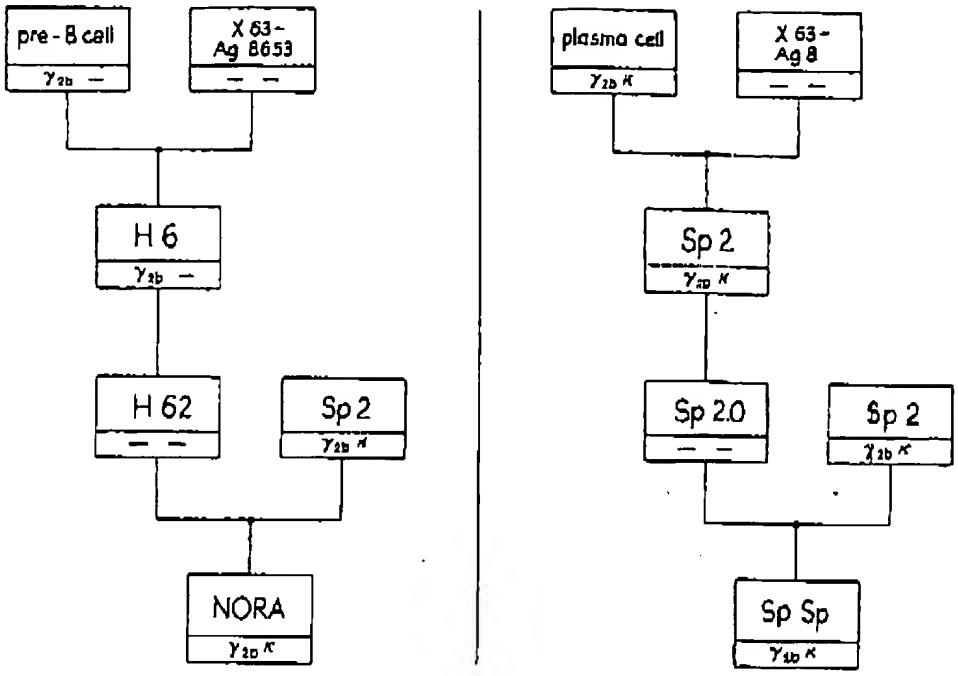

FIG. 1. Genealogy of selected hybridomas derived from a pre-B cell, or from a plasma cell, or from both. The kind of Ig chain synthesized is given below the clone designation.

ing (Fig. 3) and by immunofluorescence intensity (data not shown), was not different. This does not formally exclude that pre-B cells survive the expression of free H chain because of a low rate of synthesis, but there would still remain the question of why the H chain is not toxic for pre-B-cell-derived hybridomas as it is for plasma cell-derived hybridomas.

A Given H Chain Is Toxic in Plasma Cells but Not in Pre-B Cells. Because pre-B-cell-derived hybridomas can survive high levels of the H chain, we wanted to know whether they are able to survive the expression of a H chain, the toxicity of which has been demonstrated in plasma cells. For that purpose, we fused a plasma cell hybridoma to a pre-B-cell hybridoma that had lost its own H chain expression.

The Sp2 cell line is derived from fusion of a plasma cell with a myeloma (Fig. 1). It synthesizes both H and L chain and exhibits the H chain toxicity phenomenon (2). We confirmed this by recovering Ig chain-loss variants according to the method of Coffino and Scharff (1). Of 74 subclones, 69 expressed no H chain, one expressed no L chain (that is, expressed H chain only), and 4 did not express any Ig chain at all (Table 1). We then fused the Sp2 cell line to a pre-B-cell-derived hybridoma, H62, that had lost the expression of its own Ig chain (Fig. 1). The resulting hybrid cell line NORA 4 secreted H and L chain from the Sp2 parent line (Figs. 3 and 4). The NORA cell line tolerates the H chain in the ab-

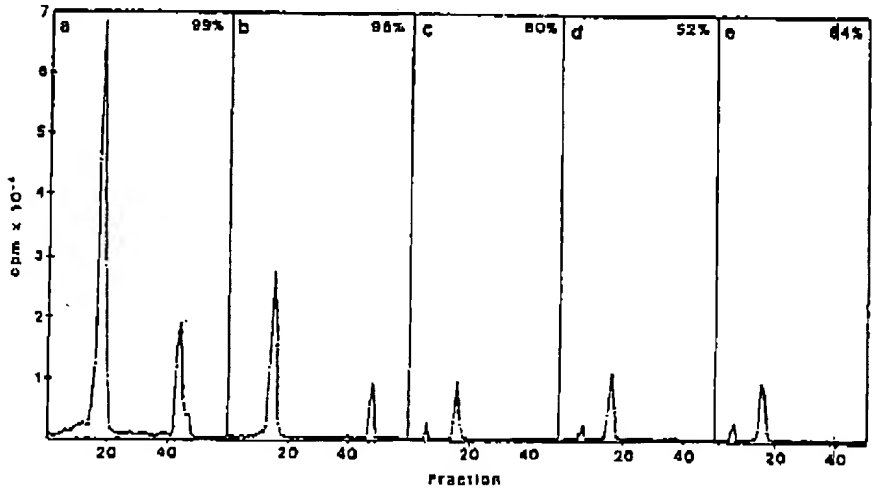

FIG. 2. Amount of radioactivity incorporated into immunoprecipitated proteins after separation on polyacrylamide gel. GK14.1 (*a*) and Sp2 (*b*) are plasma cell-derived hybridomas synthesizing both H and L chain; H32-21 (*c*), H32-3 (*d*), and H32-8 (*e*) are pre-B-cell-derived hybridomas synthesizing a H chain that is associated with BiP (11). The indicated percentages of cells synthesizing Ig were determined by immunofluorescence.

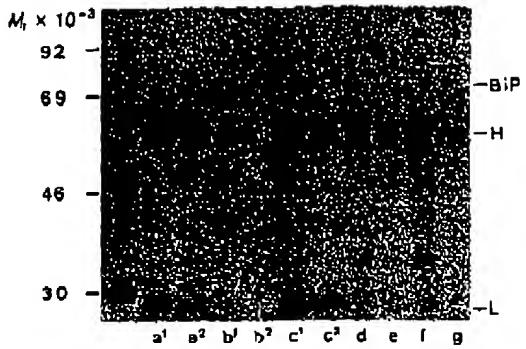

FIG. 3. NaDodSO₄/polyacrylamide gel electrophoresis analysis of the Igs of various hybridomas synthesizing both H and L chain or synthesizing the H chain only. Sp2 (lanes a) and NORA 4 (lanes c) secrete both H and L chain; SPSP 1.55 (lanes b), NORA 4.2 (lane d), NORA 4.16 (lane e), NORA 4.8.20 (lane f), and the pre-B-cell hybridoma H 61 (lane g) synthesize the H chain without the L chain but do not secrete it.

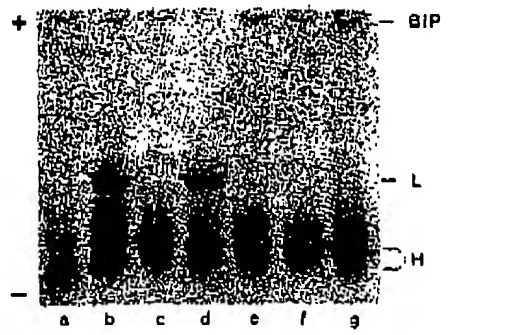

FIG. 4. Isoelectric focusing analysis of intracellular Ig of various hybridomas synthesizing both H and L chain or synthesizing H chain only. Lanes: a, pre-B-cell hybridoma H 61 (γ2b); b, Sp2 (γ2b.κ); c, SPSP 1.55 (γ2b); d, NORA 4 (γ2b.κ); e, NORA 4.2 (γ2b); f, NORA 4.16 (γ2b); g, NORA 4.8.20 (γ2b). The γ2b chain of H 61 has a variable region different from the one of the Sp2 cells and their hybridomas.

sence of the L chain. Ten out of 55 subclones of NORA 4 with Ig chain loss synthesized Ig H chain without the L chain, 42 synthesized the L chain only, and 3 did not synthesize any Ig chain at all (Table 1). A subclone of NORA 4, NORA 4.8, that synthesizes both H and L chain, showed the same chain loss distribution (Table 1). In no instance was the H chain secreted. As a control to account for the increased chromosome numbers of the NORA 4 hybrid as compared with Sp2, we fused the Sp2 cell line to a nonproducing subclone, Sp2:0, generating SPSP hybridomas (Fig. 1). From these hybridomas, subclones synthesizing free H chain should be rare. Indeed, of 125 subclones with Ig chain loss, we recovered only 1 subclone expressing free H chain (Table 1). Of these subclones, 122 had lost the H chain and two had lost both Ig chains. Comparison of the H chain of hybridomas synthesizing both H and L chain or the H chain alone revealed no difference in size (Fig. 3) nor in isoelectric focusing behavior (Fig. 4). Since in this case, the H chain is one that is known to be toxic, we conclude that in pre-B cells there is a mechanism neutralizing the toxic effect of free H chains.

DISCUSSION

Why are free Ig H chains toxic in plasma cells but not in pre-B cells? From the plasmacytoma MOPC 21, a variant could be recovered that synthesizes only the H chain. The mutant H chain of this variant lacks the last 67 carboxyl-terminal amino acids and forms polymers of at least 20 H chains (9). This polymerization is probably due to free sulfhydryl (SH) groups on the H chain that would normally form the L-H bridge. The normal H chain probably could also polymerize in the absence of L chains but would form much larger insoluble complexes that would damage the cell. Polymerization of L chains cannot occur because no additional free SH groups are available once the L chain has formed a dimer. It is of interest that H chains are predominantly found as monomers or dimers in pre-B cells (11), as well as in the H chain-synthesizing subclones of the NORA 4 hybrid line (data not shown). Thus, neutralization of the toxic effect of the free H chain may be achieved by the prevention of H chain polymerization—for example, by an enzyme that alters the reactive SH groups of the Ig H chain. Since we did not find any difference in the isoelectric focusing pattern of the intracellular H chains of Sp2, NORA 4, and of those subclones synthesizing no L chain (Fig. 4), one can postulate a protein that either rapidly degrades accumulating free H chain or protects the reactive SH groups without chemical modification. We have already described a protein (heavy chain-binding protein, BiP) that binds to Ig H chains not associated with the L chain (11). In all NORA subclones that have lost the L chain (some of them are shown in Fig. 3), the H chain is associated with the BiP. If the BiP is neutralizing H chain toxicity it should be less active in plasma cells than in pre-B cells.

Whatever the mechanism for neutralization of H chain toxicity, our results demand an explanation of why H chain synthesis precedes L chain synthesis in B-cell ontogeny (10).

Table 1. Ig chain loss pattern in various hybridomas

Hybridoma	No. of subclones		
	H chain lost	L chain lost	H + L chain lost
Sp2	57	0	4
Sp2.68	12	1	0
Total	69	1	4
NORA 4	42	10	3
NORA 4.8	42	12	0
Total	84	22	3
SPSP 1	59	1	2
SPSP 2	63	0	0
Total	122	1	2

We thank Drs. J. Johnson (Munich) and C. Steinberg (Basel) for discussions.

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0022-1767/86/1377-2367$02.00/0
The Journal of Immunology

Vol. 137, 2367–2373, No. 7, October 1, 1986
Printed in U.S.A.

GENE TRANSFER OF IMMUNOGLOBIN LIGHT CHAIN RESTORES HEAVY CHAIN SECRETION[1]

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Several lines of evidence suggest that immunoglobulin (Ig) light (L) chain plays a role in the secretion of heavy (H) chain. For example, myeloma variant lines, which synthesize the Ig H chain but not the L chain, fail to secrete H chain protein. Here we have tested directly the role of chain assembly in the control of Ig secretion by the transfer of functional L chain genes into two such L chain-defective myeloma mutants. A λ2 or κ L chain gene was introduced into variant lines of the mouse myelomas MOPC 315 (IgA, λ2) or PC7 (IgM, κ), respectively. Although the two mutant lines are unable to secrete the H chain they produce, rescue of secretion of complete Ig protein molecules (IgA or IgM) was observed after transfection. These results imply that the secretory apparatus of these cells is intact and that the failure to secrete free H chain reflects a structural feature intrinsic to that protein. The implications of these results with respect to control of secretion of multi-subunit proteins are discussed.

The immunoglobulin (Ig) heavy (H) and light (L) chains are assembled and processed intracellularly and then secreted together as a mature, functional protein (1). A normal L chain can usually be secreted in the absence of H chain. For example, Bence-Jones proteins are derived from human myelomas that secrete free L chain (2), whereas in the mouse system, there exist mutant myeloma and hybridoma cell lines that do not produce H chain but continue to secrete L chain (3). The situation for the normal H chain is different from that of the L chain. Mutant cell lines have been described that synthesize a full length H chain but no L chain (3, 4). These H chains are not secreted. Construction of hybrid lines between H chain and L chain variant lines results in H chain secretion in the form of assembled Ig molecules (5, 6). Although these results suggest that the normal full-length H chain can be secreted only when assembled with the L chain, other explanations are possible. For example, the L chain-nonproducing cell lines might have undergone a second, unrelated mutation that reduces their capacity to secrete Ig. We show here that the first hypothesis is correct, in that for two cell lines that produce H chain only, the transfer of a functional L chain gene restores H chain secretion.

Received for publication November 30, 1985.
Accepted for publication June 26, 1986.
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[1] This work was supported by grants to M. J. S. from the Allstate Foundation, The Arthritis Society (Canada), The Medical Research Council (Canada), and The Wellesley Hospital Research Institute, and to G. E. S. from the National Institutes of Health (AI 16051 and CA 36355). V. H. P. has been supported by National Institutes of Health Training Grant T32 HL 07052.
[2] Current address: Department of Immunology, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S1A8, Canada.

MATERIALS AND METHODS

MOPC 315 cell growth conditions and measurement of Ig polypeptide synthesis and secretion. MOPC 315 cells were grown and maintained by serial passage in Dulbecco's modified Eagle's medium (DME) supplemented with 16% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mM nonessential amino acids, 4 mM glutamine, and 40 mM glucose.

To assess intracellular Ig L and H chain synthesis, cultures were pulse labeled for 20 min with a mixture of six radiolabeled amino acids: 20 μCi/ml of [^{3}H]leucine, [^{3}H]lysine, [^{3}H]proline, [^{3}H]phenylalanine, [^{3}H]tyrosine, and 20 μCi/ml of [^{35}S]methionine. Washed cells were resuspended in 50 mM Tris-HCl, 50 mM NaCl and were lysed by the addition of Triton X-100 and sodium deoxycholate to 1% final concentration each, as described (6). After removal of nuclei by centrifugation at 2000 rpm for 10 min, samples of cell cytoplasmic lysates were removed for determination of trichloroacetic acid-insoluble radioactivity. Equal aliquots of cell lysates were immunoprecipitated with rabbit anti-mouse IgA protein and a goat anti-rabbit Ig antisera (Miles Yeda). Precipitates were dissolved in 50 mM Tris, pH 6.8, 2% SDS, where noted proteins were reduced with mercaptoethanol (0.5%). Samples were analyzed by SDS-PAGE as described (8 to 13%) (7).

To assess secretion of Ig protein, cultures were labeled for a 4-hr period with the amino acid mixture described above, and samples of the media immunoprecipitated and analyzed by SDS-PAGE as above.

PC7-derived cell lines and measurement of Ig polypeptide synthesis and secretion. The cell line PC7 secretes IgM specific for the hapten phosphorylcholine (PC)[3] (4). From this cell line, we isolated the mutant 574, which lacks the gene for PC-specific L chain (M. Baker, personal communication). Mutant 574 produces, but does not secrete, an apparently normal μ chain. The mutant cell line 400 lacks the μ gene of the PC7 parent line (unpublished results); it continues to synthesize the κ L chain.

PC7 cells were incubated in [^{14}C]leucine to label intracellular and secreted Ig. The μ and κ chains were precipitated by reacting with a mixture of rabbit anti-μ and anti-κ sera (Daymar Laboratories). Non-reduced samples were resolved on 4% polyacrylamide gels containing 0.1 M Tris-bicine pH 8.3, 0.1% SDS, and 0.7% *N,N'*-diallyltartardiamide as described (8). Free μ and κ chains, obtained after reduction, were analyzed with the gel system described by Laemmli (9).

Construction of vectors bearing λ2 and κ L chain genes. The 6.6-kb Eco RI genomic fragment containing the λ2 MOPC 315 L chain gene (10) was inserted into the Eco RI site of the pSV2-gpt[3] vector containing the SV40 enhancer (11), as shown in Figure 1a. The resulting vector, pGpt-λ2, kindly provided by H. Murialdo (University of Toronto), was transfected into *E. coli* strain HB101.

The 7-kb S107 fragment (12), encoding the κPc L chain, was inserted into the Bam HI site of the plasmid pSV2-neo (13). The recombinant plasmid, pNeo-κPc, containing the insert in the orientation shown in Figure 1b, was transfected into *E. coli* strain K803.

Protoplast fusion. Fusion of bacterial protoplasts with myeloma

[3] Abbreviations used in this paper: PC, phosphorylcholine; HAT, hypoxanthine-aminopterin-thymidine; gpt, guanine phosphoribosyl transferase.

EXHIBIT C

cells was performed as described (14, 15). A suspension of bacterial cells transformed with the plasmid, which had been amplified overnight in the presence of chloramphenicol, was centrifuged at 3000 rpm for 30 min, washed once with 0.9% NaCl, and resuspended in 0.05 M Tris, pH 8, 20% sucrose at a final concentration of 1×10^{10} cells/ml. To 1 ml of bacterial suspension, 0.2 ml lysozyme (1 mg/ml in 0.25 M Tris, pH 8.0) was added, and the mixture was incubated for 5 min on ice. After subsequent addition of 0.4 ml of 0.25 M EDTA, pH 8.0, the mixture was again incubated for 5 min on ice, then diluted with 0.4 ml 0.05 M Tris, pH 8.0, and incubated at 37°C for 30 min with occasional mixing. The suspension was diluted with 10 ml of medium containing 10% sucrose and 0.0125 M $MgCl_2$; 0.1 ml of 1 mg/ml DNase was added, and the mixture was incubated at room temperature for 15 min. The protoplasts (1×10^9 cells) were pelleted, supernatant was removed, and 10^7 myeloma cells were layered on top by centrifugation. The protoplast and cell mixture was gently resuspended in 1 ml PEG fusion buffer (0.85 gm PEG 1000, 1 ml DME, 0.2 ml dimethyl sulfoxide). After a 15-sec exposure, the cell suspension was diluted with DMEM without serum, and cells were pelleted by centrifugation, resuspended in medium, and distributed in microtiter wells. For the transfer of the λ2 gene, cells were placed after 3 days in HAT (hypoxanthine-aminopterin-thymidine) selective medium and then cloned in soft agar. For the transfer of the κpc gene, cells were plated at 10^4 and 10^5 cells/ml in multiwell dishes; after 2 days of incubation, the medium was supplemented with 1 mg/ml of the antibiotic G418 (13).

RESULTS

Synthesis and secretion of IgA protein by MOPC 315 LV-1 variant after λ2 L chain gene transfer. LV-1 was originally isolated as a nonsecreting variant of the mouse myeloma MOPC 315 (IgA, λ2) tumor (16). It was adapted to growth in tissue culture and was subsequently demonstrated to have lost the ability to synthesize the λ2 L chain (6). LV-1 synthesizes an altered α H chain, approximately 3000 daltons smaller than the wild-type chain. This altered H chain is not secreted, but instead is degraded intracellularly with a half-life of approximately 30 min (6). To facilitate isolation of transfectant colonies of LV-1 cells, a λ2 L chain gene cloned in the vector pSV2-gpt and an LV-1 derivative defective in hypoxanthine phosphoribosyl transferase (HPRT) activity were employed (Fig. 1a). The pSV2-gpt vector bears the bacterial gene for guanine phosphoribosyl transferase (gpt) and confers on HPRT⁻ cells the capacity to grow in HAT-containing medium (11). The cloned DNA was transferred by protoplast fusion to the LV-1 derivative line defective in HPRT activity, as described in *Materials and Methods*. Five HAT-resistant transformants were isolated and recloned for study. The individual lines were tested for λ2 L chain synthesis, and the levels were compared with the parental LV-1 line, as negative control, and with a hybrid line, HL-2, derived by fusion of LV-1 with V-1, a λ2 L chain-producing variant of MOPC 315, described previously (17). The results are displayed in Fig. 2A. The profile seen with HL-2 represents approximately wild-type amounts of λ2 and α chain synthesis for the MOPC 315 myeloma line. (The additional bands of immunoprecipitated material are the translation products of aberrant α H chain RNA species transcribed from the excluded α H chain constant region allele.) (17,18) The level of λ2 chain production by the transformants varied, but was always much lower than wild-type cells. (Values between 10- and 50-fold lower were obtained, data not shown.) The pattern from the transformant that produces the highest level of λ2 chain TLH-29 is shown in Figure 2A. As expected, n synthesis of L chain was detectable with LV-1, as observed previously (6).

The ability of the TLH-29-transfected cell line to secrete Ig was analyzed (Fig. 2B). Both α chain and λ2 chain are secreted by TLH-29, whereas no secreted Ig is observed in the media of the parental line LV-1, as mentioned above and reported previously (6). The level of secreted IgA by TLH-29 is significantly lower than that from HL-2 and is commensurate with the lower level of λ2 chain produced intracellularly by the transfected line. The band visible above the λ2 chain in the TLH-29 and HL-2 lanes has been identified as the J chain by specific immunoprecipitation (7). This protein is involved in the formation of multimeric forms of IgA protein (19) and is only secreted attached to α chain (20, 21). The other transfected lines also secreted IgA protein, although again the levels were lower commensurate with the amount of λ2 chain production (data not shown).

Although the λ2 chain of IgA is not disulfide bonded to the H chain and migrates separately in an SDS gel system, the formation of H chain multimers containing more

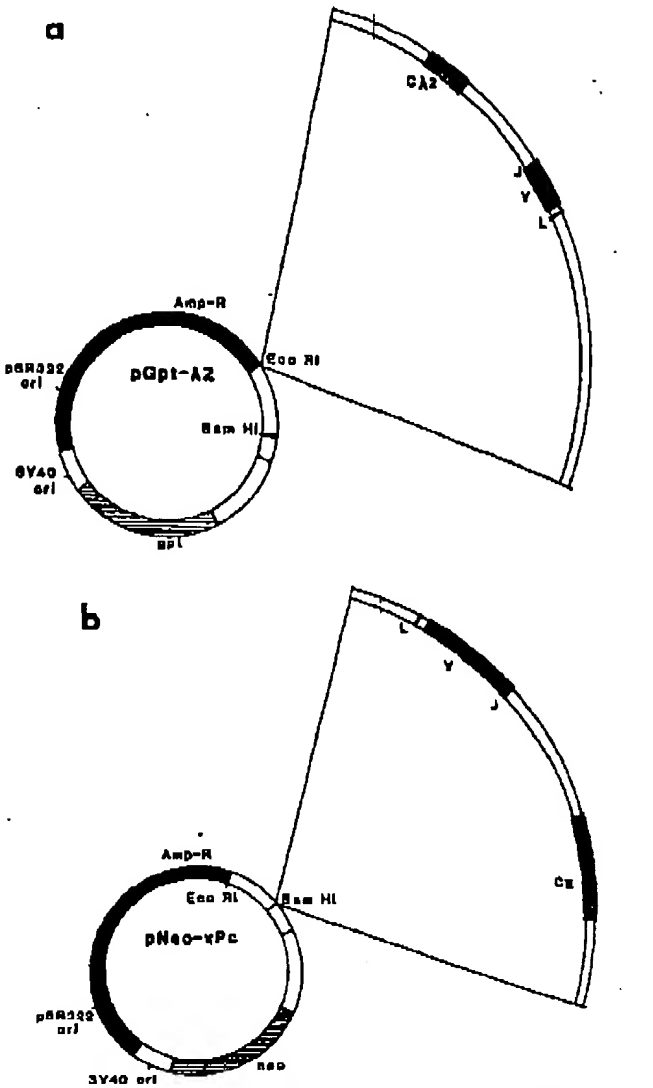

Figure 1. Construction of vectors bearing λ2 and κ L chain genes. a. The 6.6-kb cloned MOPC 315 L chain gene (10) was inserted into the Eco RI site of the pSV2-gpt vector. b. The 7-kb S107 fragment (12), encoding for the κPc L chain, was inserted into the Bam HI site of the plasmid pSV2-neo.

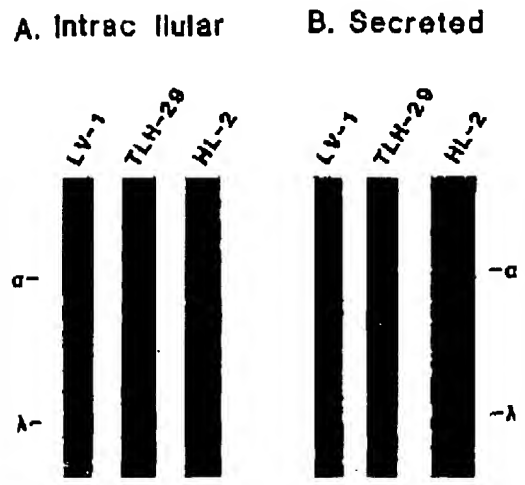

Figure 2. Rescue of Ig H chain secretion after transfer of λ2 gene. Bacterial protoplasts of strain HB101 bearing the plasmid pGpt-λ2 (Fig. 1a) were fused with LV-1. After 3 days, cells expressing the *gpt* gene were selected in HAT medium and cloned in soft agar. *A.* Analysis of intracellular Ig L and H chain synthesis. Cultures were pulse labeled for 20 min with a mixture of six radiolabeled amino acids: 20 μCi/ml of [^{3}H]leucine, [^{3}H]lysine, [^{3}H]proline, [^{3}H]phenylalanine, and [^{3}H]tyrosine and 20 μCi/ml of [^{35}S]methionine. Cell lysates were immunoprecipitated with rabbit anti-mouse IgA protein and a goat anti-rabbit Ig antisera. Precipitates were dissolved in 50 mM Tris, pH 6.8, 2% SDS, proteins were reduced with mercaptoethanol (0.5%), and equal samples were analyzed by SDS-PAGE (8 to 13%). *B.* Analysis of secreted Ig proteins. Cultures were incubated in the presence of the radiolabeled amino acid mixture for a 4-hr period; fractions of the media were immunoprecipitated and analyzed by SDS-PAGE as above.

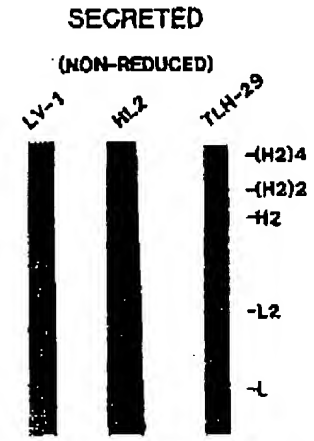

Figure 3. Structure of secreted IgA molecules after transfer of λ2 gene. Secreted IgA protein was radiolabeled and immunoprecipitated as described in Figure 2. Precipitates were dissolved in 50 mM Tris, pH 6.8, 2% SDS, and samples were analyzed by SDS-PAGE (8 to 13%) without reduction.

than two H chain molecules is diagnostic of assembled IgA (21). The mobility of the secreted Ig protein under conditions where disulfide bonds were not reduced indicated the formation of dimers and tetramers of H chain, consistent with normal multimeric IgA formation (Fig. 3). Thus, although suggestive, SDS-PAGE cannot be used to prove the formation of complete multimeric IgA forms even though, as seen below, pentameric IgM is secreted from μ chain transformants.

The kinetics of Ig protein secretion from TLH-29 were measured by pulse-chase analysis. The time course of appearance of IgA into the medium is shown in Figure 4.

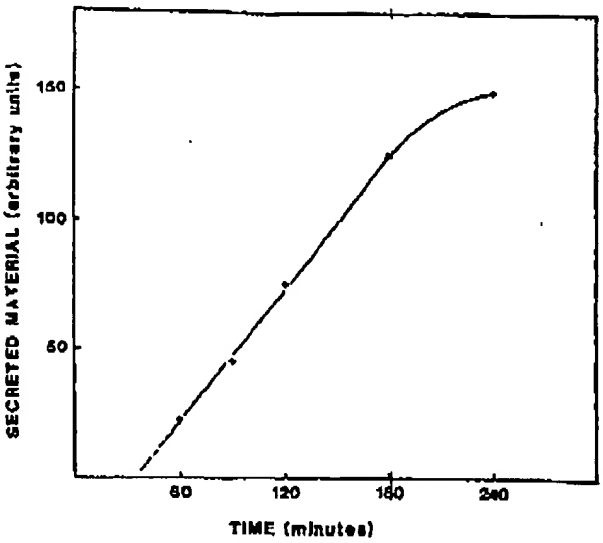

Figure 4. Kinetics of H chain secretion from LV-1 after transfer of the λ2 L chain gene. Cultures of TLH-29 were pulse labeled for 20 min as above, then washed and resuspended in fresh, warm medium. After the indicated times of chase, samples were removed and the media were analyzed for secreted α chains, as indicated in Figure 2. The autoradiograms were quantitated by scanning densitometry and integration of peak areas.

By extrapolation, secretion of newly synthesized IgA occurs after approximately 30 to 40 min. Similar values have been obtained for wild-type MOPC 315 cells (22). Thus, the time for secretion of IgA in the transformant TLH-29 line is approximately normal. Furthermore, examination of the profiles of labeled intracellular proteins indicates that expression of λ2 light chain in LV-1 cells results in stabilization of the H chain protein from degradation. The altered H chain polypeptide synthesized in LV-1 cells normally turns over with a half-life of approximately 30 min (6), and thus cannot be detected after the 240-min chase period (Fig. 5). In contrast, the kinetics of clearance of intracellular IgA from TLH-29 are similar to those observed with wild-type MOPC 315 cells (6, 22) and with the hybrid HL-2 line (Fig. 5). Thus, stabilization of the H chain from the normally rapid turnover observed in LV-1 cells occurs after expression of the introduced L chain gene.

Rescue of IgM secretion after κ L chain gene transfer. The hybridoma cell line PC7 produces IgM(κ), which is specific for the hapten PC. Using a suicide selection method (4), we obtained a mutant, 574, which produces a μ H chain that it does not secrete. From the results of Gearhart et al. (23), the PC7 cell line is expected to express the same germ-line PC-specific variable genes as are expressed in the myeloma S107. Accordingly, the κ gene cloned from S107 (Fig. 1*b*) (12) was transferred into the mutant 574 cell line. This vector carries the *neo* resistance gene, allowing for selection with the antibiotic G418 (12).

Twenty G418-resistant colonies were initially screened, of which 13 (65%) were positive for PC hemolysis (performed as described in Table I). The PC-specific lysis activity in the culture medium of these transformants ranged from nil up to the normal level. Of these, two were chosen (T574/κpc-2 and T574/κpc-10), which showed approximately normal activity, and one (T574/

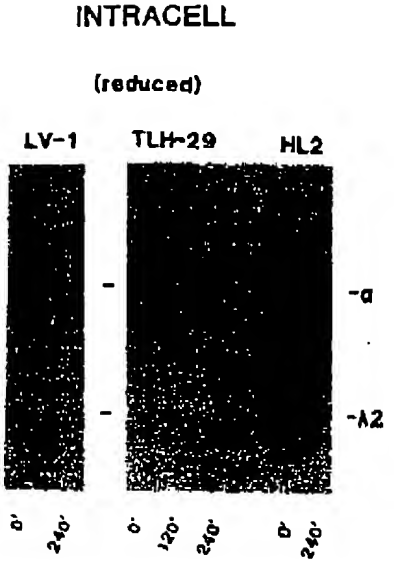

Figure 5. Pulse-chase analysis of intracellular IgA protein within LV-1 after transfer of the λ2 L chain gene. Cultures of TLH-29 were pulse labeled for 20 min as above, washed, and resuspended in fresh, warm medium. Samples, removed at the indicated times, were analyzed by immunoprecipitation and SDS-PAGE, as described in Figure 2, for L and H chains. Cultures of LV-1 and the hybrid line HL-2 were similarly treated; results for the 20- and 240-min time points are shown for comparison.

TABLE 1

Functional activity of IgM secreted by transformants[a]

Cell Lines	Hemolysis Titer	
	PC specific	μ specific
Wild type (PC7)	3[3]	3[3]
Mutant (574)	0	0
Mutant (400)	0	0
Transformants		
T574 (κpc-2)	3[3]	3[3]
T574 (κpc-10)	3[3]	3[3]
T574 (κpc-11)	0	0

[a] Culture supernatants were prepared by growing 10^6 viable cells/ml for 24 hr at 37°C. Supernatants of the cultures were threefold serially diluted, undiluted supernatant being the most concentrated material. Hemolysis titer was scored as the dilution at which a 2-μl sample caused hemolysis [4]. IgM concentration was measured by the capacity of the culture supernatant to lyse protein A-coupled red cells in the presence of anti-μ serum (4, 24) (μ-specific hemolysis titer). PC-specific hemolysis was assayed directly on red cells coupled with PC-ONS ester (4).

κpc-11) with no activity (Table I). It should be noted that the precision of the hemolysis assay is about 1 dilution step (threefold).

The analysis of intracellular preparations (Fig. 6A) indicates that the parental and transformant cell lines synthesize the μ H chain. As expected, the transformants positive for PC-specific hemolysis synthesize the L chain: conversely, the transformant that was negative for PC-specific hemolysis showed no detectable intracellular κ L chain. The level of κ chain production is consistent with the hemolysis values within the accuracy of the assay. For comparison, results have been included for the PC7 mutant cell line 400, which lacks the μ gene but continues to synthesize the κ L chain at a very low level. Secreted IgM is analyzed in Figure 6B; the transformants that make κ chain also secrete μ H chain. The mobility of the material where disulfide bonds were not reduced indicates that th IgM is secreted in th pentameric form (Fig. 6C).

The low frequency of myeloma and hybridoma mutants making only H chain has suggested that high-level production of normal H chain in the absence of L chain might often be toxic (25). Our results to date suggest that the μ H chain of mutant 574 is normal. As shown in Figure 6, it is of normal size and can be incorporated into pentameric IgM. To assess its PC binding and complement activity capacity more quantitatively, we measured its hemolytic titer on PC-coupled red cells. The IgM concentration was measured by titering its capacity to induce lysis of protein A-coupled red cells in the presence of anti-μ serum (4) (Table I). These results indicate that the IgM made by L chain transformants of mutant 574 has normal activity. We are therefore unable to detect any functional (Table I) or any structural difference (Fig. 6) between these IgM molecules and conclude that the mutant 574 makes a normal μ H chain.

DISCUSSION

Assembly plays an important role in the secretion of Ig protein. Here we have demonstrated that the introduction of an appropriate λ or κ L chain gene restores secretion of α or μ H chain as assembled IgA or IgM molecules. These results directly demonstrate that L chain production is normally required for a cell to secrete H chain. This result explains the rescue of H chain secretion by myeloma H chain-producing cells after hybrid formation with L chain-producing cells, as well as the failure of investigators from several laboratories to isolate myeloma variants capable of secreting free normal H chain (5, 6, 25). This conclusion is also consistent with the observation of Valle et al. (26) that Ig H chain dimers accumulate in oocytes microinjected with myeloma H chain mRNA and are not secreted unless L chain mRNA is also injected. The finding that the selective inhibition of λ2 production in MOPC 315 by idiotype-specific T suppressor cells does not result in the secretion of free α chain (27) is also in agreement with our conclusion.

Genetic approaches have also implicated control at the level of assembly for secretion of J chain, the joining protein necessary for multimeric Ig protein formation. MOPC 315 mutants, which have lost the ability to synthesize α H chain, fail to secrete J chain protein (20). Only L chains can, in general, be secreted in the absence of H chains (2, 3, 6, 25). Some unusual L chains, the PC-specific chain (28) (Fig. 4) and the κ chain of myeloma tumor MOPC 21 (5), are secreted only when paired with a H chain. Their failure to be secreted presumably reflects some molecular feature of the variable region, a hypothesis consistent with the observation of Wu et al. (10), that the substitution in position 15 from gly to arg in the λ2 L chain of MOPC 315 blocks its secretion. It is interesting that the PC-specific μ and κ chains can be secreted together as IgM, whereas neither of these Ig chains can be secreted alone without the other.

The expression of the introduced λ2 L chain gene was very low, even though many copies (≥100) of the cloned DNA were detected in the LV-1 transfectants by Southern blot analysis (data not shown). This expression was probably driven by the SV40 enhancer, present on the pSV2-gpt vector, as was shown previously for transfection of the λ1 L chain gene (29). Alternatively, integration into a "transcriptionally open" site in the chromatin may have played a role. The inability of researchers to find a λ L

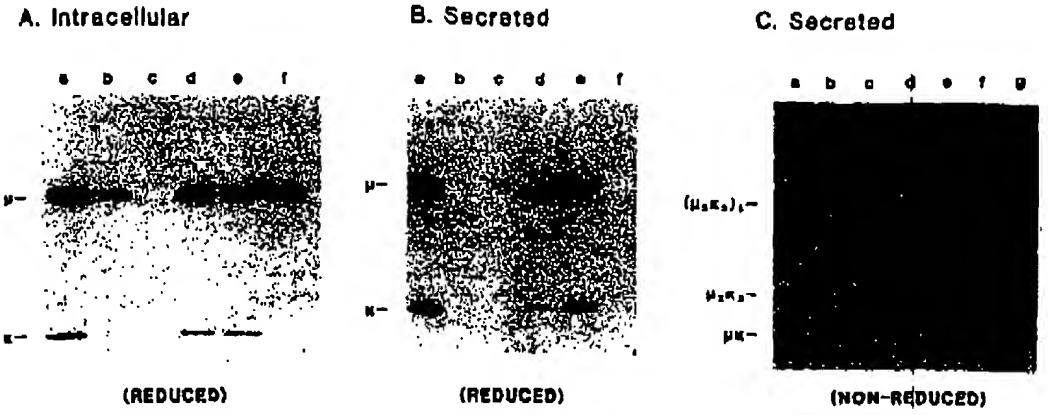

Figure 6. Analysis of Ig production after transfer of κ gene. The plasmid pNco-κPc (Fig. 1b) was introduced into the bacterium K803, and protoplasts were fused with the mutant S74 myeloma cells. Colonies (transformants) resistant to 1 mg/ml G418 were selected and recloned by limiting dilution. Cells were incubated in [^{14}C]leucine, as described (4), to label intracellular and secreted Ig. The μ and κ chains were precipitated by reacting with a mixture of rabbit anti-μ and anti-κ sera. Lanes: a) wild-type PC7; b) mutant S74; c) mutant 400; d) transformant T574/κpc-2; e) transformant T574/κpc-10; f) transformant T574/κpc-11. *A*, Analysis of intracellular material after reduction of disulfide bonds. *B*, Analysis of secreted material after reduction of disulfide bonds. *C*, Analysis of secreted material without reduction of disulfide bonds. In lane g, the mutant 21, which makes monomeric IgM (4), is included for comparison.

chain enhancer is certainly puzzling. Clearly in our experiments, the endogenous gene was expressed at a significantly higher rate than the introduced cloned version. Because the pGpt-λ2 clone encompassed 6.6 kb of the MOPC 315 λ2 L chain gene, this result suggests either that the regulatory sequences are more distant or that the endogenous gene has a different conformation, one that is better able to interact with cellular factors required for optimal λ L chain gene transcription.

Thus in a normal situation, free H chain polypeptide is not in an appropriate form to be secreted. This may be due simply to structural differences between a free H chain compared with a H chain assembled in an Ig molecule. Thorens et al. (30) have shown that free μ chains are directed via the *cis* golgi to lysosomes where they are degraded, whereas μ-L chain pairs migrate via the *trans* golgi to the cell surface. In some myeloma variants the H chain is degraded intracellularly (6); in other cases it remains within the cell (3). The results of the experiments presented here indicate that assembly of the H chain with a L chain is sufficient to restore secretion of those polypeptides in a complete Ig protein. The importance of assembly in control of secretion has also been demonstrated for collagen (31), β_2-microglobulin (32), and the T cell receptor (33). Thus, the assembly of multi-subunit proteins is often a requirement for protein secretion, and thus might serve as an important regulatory step in the secretory process.

Acknowledgments. We thank H. Murialdo for generously providing the pGpt-λ2 clone. The excellent typing of Ms. Michaelle Montgomery is gratefully acknowledged.

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INTERNATIONAL WEEKLY JOURNAL OF SCIENCE
Volume 342 No.6245 [illegible]

ANTIBODY PRODUCTION IN TRANSGENIC PLANTS

CELL BIOLOGY
product review

EXHIBIT D

(PCT 195) 37 Nature
342(1989)2 November, no.6245, London, GB
XP 000069445
C12N 15/82

mechanism of NA release from rat cerebral-cortex synaptosomes. Because immunocytochemical studies have shown that B-50 is found in synapses throughout the brain[21,22], B-50 could be more generally involved in transmitter release. Three lines of evidence suggest that the phosphorylation of B-50 by PKC is essential for stimulus–secretion coupling during transmitter release: (1) phorbol esters that directly activate PKC enhance the release of a variety of neurotransmitters[1-4]; (2) by using an antibody-independent approach, we have previously shown that depolarization-induced neurotransmitter release from non-permeabilized synaptosomes and hippocampal slices is closely correlated with a PKC-mediated increase in B-50 phosphorylation[16,23]; and (3) here we have shown that anti-B-50 IgG inhibits B-50 phosphorylation as well as Ca^{2+}-dependent transmitter release. If B-50 phosphorylation by PKC is indeed involved in the mechanism of transmitter release, then a long-term increase in PKC-mediated B-50 phosphorylation[7,17] could be one of the mechanisms underlying the increase in the release of glutamate that occurs during long-term potentiation[5-7,24,25].

In view of the localization of B-50 at the inner leaflet of the plasma membrane[21,26], we suggest that B-50 is involved in the regulation of vesicle fusion with the plasma membrane, a process in which the vesicle-associated protein synapsin I (a substrate of calmodulin-dependent kinases) has also been implicated[27,28]. But the difference in the localization of phosphorylating enzymes of these two proteins indicates that they have distinct roles in the transmitter release process. It may be that the regulatory role of B-50 in vesicle fusion is not limited to transmitter release, but extends to membrane-fusion processes during neurite outgrowth[29,30]. It remains to be investigated to what extent calmodulin binding[14] and modulation of phosphatidyl inositol 4-phosphate kinase activity[12,13]—putative properties of B-50—are also involved in controlling neurotransmitter release. ☐

Received 17 April; accepted 11 September 1989.

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Production of antibodies in transgenic plants

p.76-78

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COMPLEMENTARY DNAs derived from a mouse hybridoma messenger RNA were used to transform tobacco leaf segments followed by regeneration of mature plants. Plants expressing single gamma or kappa immunoglobulin chains were crossed to yield progeny in which both chains were expressed simultaneously. A functional antibody accumulated to 1.3% of total leaf protein in plants expressing full-length cDNAs containing leader sequences. Specific binding of the antigen recognized by these antibodies was similar to the hybridoma-derived antibody. Transformants having γ- or κ-chain cDNAs without leader sequences gave poor expression of the proteins. The increased abundance of both γ- and κ-chains in transformants expressing assembled gamma-kappa complexes was not reflected in increased mRNA levels. The results demonstrate that production of immunoglobulins and assembly of functional antibodies occurs very efficiently in tobacco. Assembly of subunits by sexual cross might be a generally applicable method for expression of heterologous multimers in plants.

The source of immunoglobulin mRNAs was a hybridoma cell line expressing a catalytic IgG_1 antibody (6D4) which binds a low molecular weight phosphonate ester (P3) and catalyses the hydrolysis of certain carboxylic esters. Constructs used for immunoglobulin expression in plants consisted of coding-length cDNAs of the 6D4 γ- or κ-chain with or without their leader sequences. These cDNAs were modified to contain terminal *Eco*RI restriction enzyme digestion sites and were ligated into the constitutive plant expression vector pMON530 (ref. 2) to form pHi101 (kappa, no leader), pHi102 (kappa, leader), pHi201 (gamma, no leader) and pHi202 (gamma, leader). We transformed tobacco plants using Agrobacterium containing each of these four plasmids[3] and screened leaf extracts from regenerated transformants for the presence of immunoglobulin heavy or light chains by enzyme-linked immunosorbent assay (ELISA)[4]. Transformants expressing individual immunoglobulin chains were then sexually crossed to produce progeny expressing both chains. The results of the ELISA revealed high levels of kappa and gamma chains accumulating in individual plants containing DNA from both pHi102 and pHi202 (Table 1; Fig. 2a). We verified the expression of both heavy and light chains by western blotting (Fig. 1). From the ELISAs, we judged that virtually all the γ- and κ-chains in these plants were assembled into gamma–kappa complexes (Table 1). Western blots provided additional evidence for assembled antibodies in that, under non-reducing conditions, most of the immunoreactive γ- and κ-chains aggregated at a high molecular weight (Fig. 1).

The binding specificity of the assembled gamma–kappa complexes was studied in ELISAs in which a P3–bovine serum albumin conjugate was used as antigen. The antigen binding by antibody derived from plants was equivalent to antigen binding by the 6D4 hybridoma antibody. Incubation of plant extracts or the purified 6D4 antibody with 50 μmol l^{-1} P3 for 3 h at 25 °C before addition to the ELISA eliminated antibody binding to the P3–BSA conjugate, demonstrating that binding was specific for the P3 hapten. Half-maximal inhibition occurred with 10 μmol l^{-1} free P3 for both hybridoma and plant-derived antibodies.

Transformants derived from the leaderless constructs pHi101 and pHi201 contained very low levels of κ- and γ-chains respectively, but Southern and northern blots (Fig. 2) demonstrated the presence of transforming DNA and immuno-

immunoglobulin chains contained assembled gamma-kappa complexes (Table 1).

The increased recovery of immunoglobulin epitopes from transformants expressing full-length cDNAs was not reflected in increased mRNA transcript levels. Northern blots (Fig. 2b) comparing pHi201 and pHi202 transformants, for example, revealed nearly equivalent levels of heavy-chain transcripts, although ELISAs indicated a 40-fold increase in accumulation of heavy-chain protein in the pHi202 transformant. Likewise, immunoglobulin mRNA levels in a plant producing large amounts of assembled antibodies were not significantly different from the parental plants that accumulated low levels of immunoglobulin chains (Fig. 2b).

Our results show that individual cDNAs for immunoglobulin κ- and γ-chains can be efficiently expressed in tobacco to form functional antibodies. Assembly of immunoglobulin chains by sexual cross in plants represents a useful alternative to the expression by a single vector of both gamma and kappa cDNAs as in yeast or bacteria[3,4], or double transformation with vectors containing individual cDNAs[5,6]. Potentially, this method is applicable to the assembly of oligomers other than antibodies. The characterization of antibodies produced in plants (glycosylation, processing of leader sequences, cytolocalization and turnover) will be described in a later paper.

In B lymphocytes, immunoglobulin processing and assembly occurs in the endoplasmic reticulum/Golgi in a process that may be promoted by heavy-chain binding protein present in the endoplasmic reticulum[10,11]. Plant cells may also have a system for multimer assembly in their endoplasmic reticulum/Golgi that can recognize immunoglobulin chains. Alternatively, assembly may occur spontaneously, given sufficient levels of each chain in the appropriate cellular compartment. Our results demonstrate that plants require a signal sequence for efficient assembly of γ- and κ-subunits. The presence of the mouse leader sequence clearly augments the accumulation of individual chains. This might be the result of an enhanced translation of the immunoglobulin messengers or an increased stability of each protein as a result of subcellular sequestering or secretion. The yield of each chain is increased in plants expressing both gamma and kappa, indicating that assembly of the gamma-kappa complex might enhance stability.

TABLE 1 Expression and assembly of immunoglobulin gamma and kappa chains in tobacco

Accumulation of γ- or κ-chains in transformed plants*

γ^{NL}	γ^{L}	$\gamma^{L}(\kappa^{L})$	$\gamma^{NL}(\kappa^{NL})$
30 ± 16 (60)	1,412 ± 270 (2,400)	3,330 ± 2,000 (12,800)	32 ± 26 (60)
κ^{NL}	κ^{L}	$\kappa^{L}(\gamma^{L})$	$\kappa^{NL}(\gamma^{NL})$
1.4 ± 1.2 (3.5)	58 ± 5 (80)	3,700 ± 2,300 (12,800)	6.5 ± 5 (20)

Distribution and assembly in crosses†

	γ only	κ only	$\gamma\kappa$	Null
$\kappa^{NL} \times \gamma^{NL}$	4	8	3 (0% assembly)	5
$\kappa^{L} \times \gamma^{L}$	3	10	11 (95 ± 16% assembly)	4

* Accumulation of individual gamma and kappa chains (in ng per mg total protein) was estimated by ELISA[8]. Microtitre wells were coated with a goat anti-mouse heavy or light chain-specific IgG (Fisher) in 150 mM NaCl, 20 mM Tris-HCl, pH 8.0 (TBS), followed by blocking with 5% non-fat dry milk in TBS. Plant leaves were homogenized in a mortar and pestle at 4 °C after removal of the midvein. To the supernatant a quarter volume of 5 × TBS was added, and 50 µl of 1 in 2 serial dilutions were added to each microtitre well. After 18 h at 4 °C, microtitre wells were washed with distilled water at room temperature. Bound γ- or κ-chains were reacted with goat anti-mouse heavy or light chain-specific antibodies conjugated to horseradish peroxidase for 2 h at 37 °C in TBS, and detected according to the manufacturer's instructions. Control microtitre wells contained extracts from plants transformed with pMON530 vector. Values given as mean (± s.d.) are derived from at least two determinations per plant and do not include transformants producing no detectable γ- or κ-chain. At least nine plants were assayed in each category. All values are given as ng per mg of total protein in the extract and are derived from the quantity of purified 6D4 antibody required to given an equivalent colour development in ELISA. Total protein in the extract was determined by the Bio-Rad Coomassie assay. Complementary DNAs containing no leader sequences are referred to as γ^{NL} and κ^{NL}; γ^{L} and κ^{L} refer to cDNAs with leader sequences; $\gamma(\kappa)$ refers to gamma chains in a plant that also expresses κ-chains, and vice versa. Numbers in parentheses are values for plants with the highest levels of accumulation.

† The number of plants expressing γ- or κ-chains among the progeny of a sexual cross. The ELISA for assembly used horseradish peroxidase-conjugated anti-κ-chain-specific antibodies to detect antigen bound to microtitre wells coated with unlabelled anti-γ-chain-specific antibodies, and vice versa. Values derived from these assays were used to calculate the per cent of assembly by comparison with the purified 6D4 antibody. This was determined at least three times for each $\gamma\kappa$ plant. The per cent assembly is expressed in parentheses as the mean ± s.d.

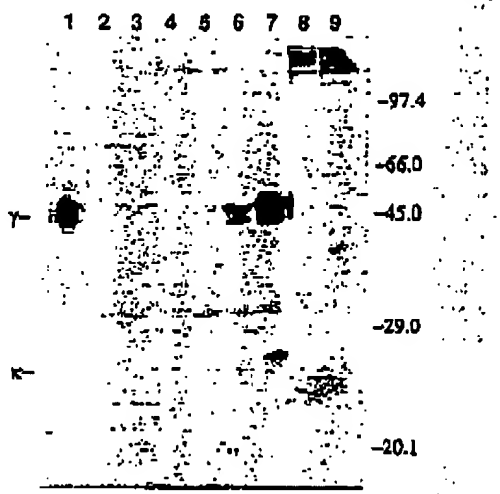

FIG. 1 Western blot of leaf proteins from transgenic tobacco plants expressing immunoglobulin chains. Leaf segments (1 g) from mature plants were homogenized in a mortar and pestle with 1 ml 0.05 M Tris-HCl, pH 7.5, 1 mM phenylmethanesulphonyl fluoride. Extracts were boiled in 4 M urea, 1% SDS, with or without 2 mM dithiothreitol (DTT) as indicated, for 3 min. SDS-PAGE in 10% acrylamide[16] and blotting of the proteins to nitrocellulose[17] were performed as described. Blots were preincubated for 6 h at 4 °C in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01% Tween 20 (TBST) containing 5% BSA and 0.5% non-fat dried milk before the addition of antibodies. A biotinylated goat anti-mouse whole IgG antibody (Cappel, diluted 1:500 in TBST was used to probe the blots at 4 °C for 24 h. A variety of commercially available antibodies (anti-mouse IgGa) were used in other experiments with similar results. Antibody binding was visualized after binding of streptavidin-conjugated alkaline phosphatase (25 °C, for 2 h) by incubation in 300 µg ml^{-1} nitroblue tetrazolium and 150 µg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate, in lanes 1–7, 40 µl of each extract containing DTT; lanes 8 and 9, 40 µl extract without DTT. Lane 1, 100 ng purified antibody from the 6D4 hybridoma; lane 2, 15 µg wild-type plant-extract protein; lane 3, 15 µg protein from a plant transformed with truncated κ-chain cDNA (pHi101) containing no leader sequence; lane 4, 15 µg from plant transformed with truncated γ-chain cDNA (pHi201); lane 5, 15 µg from a full-length kappa cDNA transformant (pHi102); lane 6, 15 µg from a full-length γ-chain cDNA transformant (pHi202); lane 7, 15 µg from an F1 plant derived from the cross between a kappa and a gamma producer; lane 8, 100 ng 6D4 antibody (no DTT); lane 9, same as lane 7, except no DTT. Gamma and kappa on the left refer to the positions of the 6D4 heavy and light chains; positions of molecular weight (given in thousands) markers are shown on the right. By ELISA, extracts in lanes 3–9 contained very low levels of κ- or γ-chains (<0.008% of total protein, Table 1), whereas extracts in lanes 6, 7 and 9 contained 0.24, 1.3 and 1.3% immunoglobulin respectively.

SAN DIEGO COUNTY
Los Angeles Times
THURSDAY, NOVEMBER 2, 1989

COLUMN ONE

Parents Spell the Difference

■ Their extraordinary role helped bring an end to Beverly Hills teachers strike. Educators call it a how-to manual.

By JEAN MERL
TIMES EDUCATION WRITER

When Day 3 of the Beverly Hills teachers strike dawned with no negotiations in sight, real estate investor Robert Schwab, whose children attend the city's prestigious public schools, persuaded the union and the district to go back to the bargaining table.

[illegible]

Andrew Hiatt of Scripps Clinic in La Jolla checks a petri dish for root growth in antibody-producing plant.

Scripps Technique Seen as Big Medical Stride

■ Science: Researchers develop method of producing antibodies that could open up new medical field.

By THOMAS H. MAUGH II
TIMES SCIENCE WRITER

Researchers at the Scripps Clinic in La Jolla have developed an inexpensive technique for producing specialized antibodies that promises to open up a whole new area of medicine, including cancer therapy, and to greatly expand medical diagnostics.

[illegible]

Conferees Said to Retain B-2, Cut 'Star Wars'

By JOHN M. BRODER
TIMES STAFF WRITER

WASHINGTON—House and Senate conferees Wednesday reached accord on virtually all remaining issues involving the $305-billion 1990 defense budget, trimming the "Star Wars" anti-missile program but keeping the costly and controversial B-2 Stealth bomber alive, congressional sources said.

[illegible]

Truce Is Over, Ortega Declares

■ Nicaragua: He vows to drive out the Contras. He also calls for peace talks, but their resumption is uncertain.

By RICHARD BOUDREAUX
TIMES STAFF WRITER

MANAGUA, Nicaragua—President Daniel Ortega formally ended a 19-month-old cease-fire against U.S.-backed rebels Wednesday and said the Sandinista army will try to push them back to bases in Honduras. But he asked rebel leaders to meet in New York next Monday for talks on ending the [illegible] Nicaraguan war.

[illegible]

Ortega at press conference

U.S. Condemns Move, Hopes It Will Backfire

By DAVID LAUTER
TIMES STAFF WRITER

WASHINGTON—U.S. officials denounced Nicaraguan President Daniel Ortega on Wednesday for ending the 19-month cease-fire with the Contras, but they expressed optimism that the move will backfire and [illegible].

[illegible]

EXHIBIT E

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A PENDING SECOND APPLICATION	Docket No. TSRI 184.2C2

In the application of:	Hein et al.
Serial No.:	09/200,657
Filed:	November 25, 1998
For:	TRANSGENIC PLANTS EXPRESSING ASSEMBLED SECRETORY ANTIBOEODIES

The owner, The Scripps Resesarch Institute, of 100% percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. §§ 154 to 156 and 173 as shortened by any terminal disclaimer filed prior to the grant of any patent granted on pending second Application Number 09/512,736, filed on February 24, 2000, of any patent on pending second application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the second application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successor or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend the expiration date of the full statutory term as defined in 35 U.S.C. §§ 154 to 156 and 173 of any patent granted on the second application, as shortened by any terminal disclaimer filed prior to the patent grant, in the event that any such granted patent: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 C.F.R. § 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to grant.

Check either box 1 or 2 below, if appropriate.

1. ☐ For submissions on behalf of an organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

2. ☒ The undersigned is attorney or agent of record.

Dated: December 27, 2001

By: Barry Wilson
Name: Barry S. Wilson
Registration No.: 39,431

☐ Terminal disclaimer fee of $110.00 under 37 C.F.R. § 1.20(d) is attached.

☒ Please charge the terminal disclaimer fee of $110.00 to **Deposit Account 50-0872.**

☒ PTO suggested wording for Terminal Disclaimer was:

☒ unchanged ☐ changed (explanation on attached sheet).